Application of Biochemical Biomarkers

to

aid Precision Diagnosis

By

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Declarations

I declare that the submitted material is not substantially the same as published or unpublished material that I have previously submitted, or am currently submitting, for a degree diploma, or similar qualification at any university or similar institution.

WORD COUNT: 10,610 (Excluding references)

List of publications

These publications have been submitted for consideration for the degree of Doctor of Philosophy by Published Work. A statement describing the contribution to each publication is stated, with the supporting letters provided in Appendix 1. The full papers can be found in Appendix 2.

Papers	**Statement of contribution**
Paper 1 **Chatha K**, Anderson NR, Gama R, (2002), Ethnic variation in C-reactive protein: UK resident Indo-Asians compared with Caucasians, *J Cardiovasc Risk.* 9(3):139-41.	K.Chatha contributed to the study design, processed the sample collection, test analysis, statistical analysis and manuscript.
Paper 2 **Chatha K**, Alsoud M, Griffiths MJ, Elfatih A, Abozguia K, Horton RC, Dunmore SJ, Gama R, (2006), B-type natriuretic peptide in reversible myocardial ischaemia, *Clin Pathol.* 59(11):1216-7.	K.Chatha contributed to the study design, processed the sample collection, test analysis, statistical analysis and manuscript.
Paper 3 Armitage AE, Stacey AR, Giannoulatou E, Marshall E, Sturges P, **Chatha K**, Smith NMG, Huang XJ, Xu XN, Pasricha SR, Li N, Wu H, Webster C, Prentice AM, Pellegrino P, Williams I, Norris PJ, Drakesmith H, Borrow P, (2014), Distinct patterns of hepcidin and iron regulation during HIV-1, HBV, and HCV infections, *Proc*	K.Chatha contributed to this publication by analysing all samples for the biomarkers discussed in the paper on the automated analysers **Letter 1 –** unable to obtain supporting letters from all co-authors, an email was sent to the corresponding author, but no response received.

Natl Acad Sci USA. 2014;111(33):12187-92.	
Paper 4 Kennedy DM, **Chatha K**, Rayner HC, (2013), Laboratory database population surveillance to improve detection of progressive chronic kidney disease, *J Ren Care.* 39 Suppl 2:23-9.	K.Chatha contributed to the design of the study, performing the analysis of the data and the preparation of the manuscript. **Letter 2 and 3.**
Paper 5 Puszyk WM, **Chatha K**, Elsenheimer S, Crea F, Old RW, Methylation of the imprinted GNAS1 gene in cell-free plasma DNA: equal steady-state quantities of methylated and unmethylated DNA in plasma, *Clin Chim Acta*. 400(1-2):107-10.	Letter not available – B.Old has retired and not responding to emails, W.Puszyk cannot be located. F.Crea and S.Elsenheimer cannot be contacted.
Paper 6 Anderson NR, **Chatha K**, Holland MR, Gama R, (2003), Effect of sample tube type and time to separation on in vitro levels of C-reactive protein, *Br J Biomed Sci.* 60(3):164-5.	K. Chatha helped perform the analysis and contributed to the manuscript. **Letter 4–** no supporting letters from MR Holland, who retired 20 years ago and no contact details available.
Paper 7 **Chatha K,** Middle JG, Kilpatrick ES, (2010), National UK audit of the short synacthen test, Ann *Clin Biochem.* 47(Pt 2):158-64.	K. Chatha collated the data, performed the data analysis and prepared the manuscript.

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Abbreviations

AI	Adrenal Insufficiency
ASSIST-CKD	Chronic Kidney disease quality improvement project
AIDS	Acquired immunodeficiency syndrome
BP	Blood pressure
BNP	Brain Natriuretic peptide
CHD	Coronary heart disease
Cfp	Cell-free plasma DNA
CKD	Chronic kidney disease
CLSI	Clinical and Laboratory Standard Institute
CpG	Cytosine-guanine
CRP	C-reactive protein
CVD	Cardiovascular disease
EDTA	Potassium ethylenediaminetetraacetic acid
eGFR	Estimates glomerular filtration rate
ESFR	End-stage renal failure
EQA	External quality assurance
Fpn	Ferroportin receptor
GP	General practitioner
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDL cholesterol	High-density lipoprotein cholesterol
HIV-1	Human immunodeficiency virus-1
hsCRP	High-sensitive C-reactive protein
ID-GCMS	Isotope dilution Gas Chromatography Mass Spectrometry
IFCC	International Federation pf Clinical Chemistry and Laboratory Medicine
IHD	Ischaemic heart disease
IL	Interleukin
LIMS	Laboratory Information System
NEQAS	National External Quality Assurance Scheme
NICE	National Institute for Health and Care Excellence
NT-proBNP	N-terminal pro-B-type Natriuretic Peptide
SST	Short Synacthen Test
UHCW	University Hospitals Coventry and Warwickshire
UIBC	Unsaturated iron binding capacity

WHO	World Health Organisation

Abstract

Biochemistry is a ubiquitous speciality that crosses many specialities in medicine. Biomarkers are the foundation on which testing is done in this discipline. The identification and assessment of biomarkers has been well established, but the interpretation of these biomarkers against a reference population is impacted by various factors. There are pre-analytical, analytical and post-analytical factors that need to be taken into consideration as well as the population the biomarker is validated from.

The current need for precision medicine has driven the need for precision diagnosis. The current processes to identify and interpret biomarkers using large population data, which opposes this new requirement.

This thesis presents seven papers that provide data for three areas where precision diagnosis is challenged due to the health burden of each disease. The first area is the detection of disease in high-risk patients, the second is the detection of biomarkers that detect poor prognosis in patients with chronic disease and the third is to optimise pre- and post-analytical to improve biomarker applications.

The papers here demonstrate that they meet these aims are provide important data to each individual field, ultimately, improving the clinical use of the biomarkers in improve detection of disease and initiating treatment that will reduce morbidity and mortality. As stated earlier these papers span different specialities where Biochemistry plays an important role.

1. Introduction

1.1. Clinical Biochemistry

Clinical Biochemistry is the largest sub-speciality of Laboratory Medicine, which can trace its origins back to 400 BC, when it was reported that Greeks noticed that ants were attracted to “sweet urine” [1]. Additionally, Hippocrates (460-375 BC) described a “test” that included inspecting the bubbles in urine, indicating a disease of the kidneys, which we now know to be protein in urine [2].

Since then, the role and scope of the Clinical Biochemistry laboratory has developed and expanded to embed many different analytical techniques, from spectrophotometry, immunoassay to Mass Spectrometry (MS) and molecular testing. The laboratory at UHCW analyses approximately 6 million tests per year and the activity has increased year-on-year.

Currently the Clinical Biochemistry Laboratory is used extensively across all medical disciplines in primary and secondary care. The commonly used statistic stated is that 70-80% of clinical decisions are made on laboratory tests, though the source of this figure cannot be identified [3], however, it is universally accepted that Clinical Biochemistry laboratory testing is an integral and fundamental part of patient care. The tests carried out by the laboratory includes application in diagnosis, prognosis, screening and monitoring.

1.2. Biomarkers

The use of biomarkers is often used interchangeably for the word “tests’, as such, going forward biomarkers will be used exclusively in this thesis. The definition of biomarkers is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processed, or pharmacologic responses to a therapeutic intervention [4].

1.3. Application and interpretation of biomarkers

Before a biomarker can be measured in routine clinical practice, it must show that it has some value in identifying disease conditions, and an example of the selection pathway is shown in Figure 1 below.

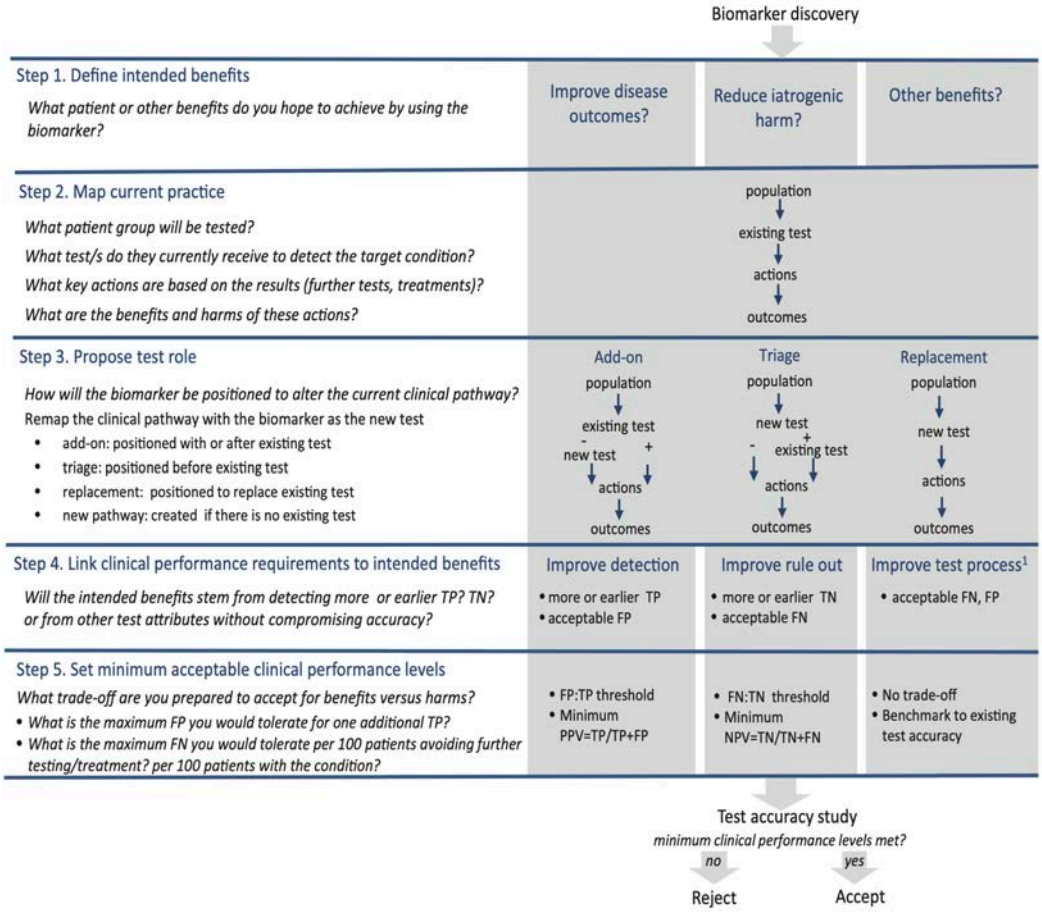

Figure 1 Guide for setting clinical specifications for biomarkers. Diagram sourced from Setting clinical performance specifications to develop and evaluate biomarkers for clinical use [5]

As with any data, the values must be appropriately interpreted to provide valid conclusions. In the case of biomarkers, the results must be interpreted against values that encompass the "healthy" or "normal" population and those that encompass the "unhealthy" or "diseased' population, which includes reference intervals, therapeutic ranges, clinical decision limits, and risk calculations. The biomarker results can then be used in conjunction with the clinical symptoms and history to make decisions that directly impact patient care.

1.3.1. Sensitivity and specificity of biomarkers

The sensitivity and specificity of biomarkers have an important impact on the accuracy of biomarkers in the clinical context, which is why the identification of reference intervals and clinical decision limits must be set at values that optimise their clinical usefulness. It is ideal that the biomarker detects disease in 100% (sensitivity) of those with the disease and excludes 100% of those without disease (specificity), however, no biomarker achieves this level of accuracy. This is because the distribution of biomarker results in diseased individuals overlaps with those that are non-diseased.

The performance of a biomarker can be identified by using Table 1, where the diagnostic value of the biomarker is compared to the reference (gold standard) result for a disease state.

	Disease status	
Test result	Diseased	Non-diseased
Positive	True positive (TP)	False positive (FP)
Negative	False negative (FN)	True negative (TN)

Table 1 Description of the performance of biomarkers against the disease state

These values can be used to identify diagnostic accuracy, sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV).

The performance of a biomarker is affected by the prevalence of the specific disease, in the population being observed, therefore, the NPV and PPV can vary even with high sensitivity and specificity [6].

1.3.2. Reference interval

The development and calculation of reference interval is now a well-established process for biomarkers. There is a need to consider biological and analytical variation in order to establish a reference interval and are a representation of 95% of the healthy population. As laboratory medicine developed, approaches to identifying reference interval were described by the IFCC and CLSI in several guidelines and publications [7-10], see Figure 1.

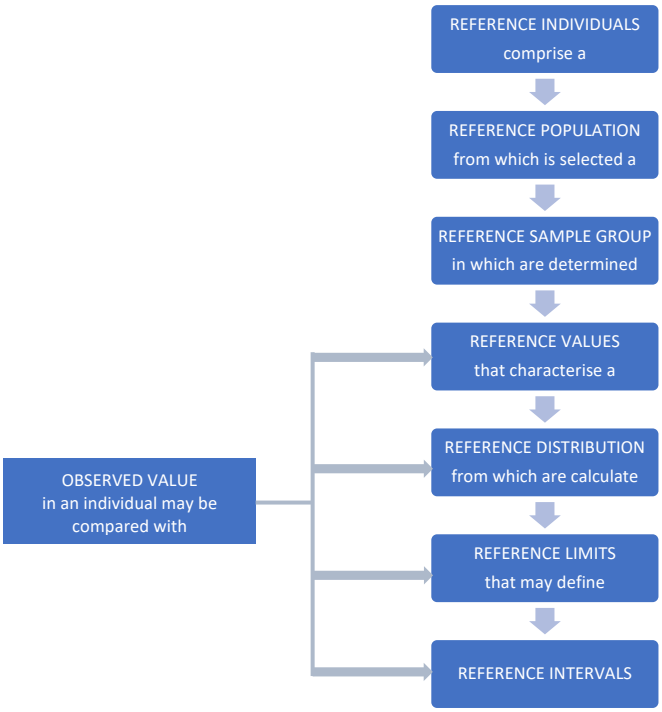

Figure 2 Description of the stages and terms related to development of reference intervals as stated by IFCC and CLSI. Diagram adapted from IFCC recommendation-theory of reference values. [10]

There are two approaches identified either the direct sampling technique or the indirect sampling technique in order to select a cohort of patients to develop reference intervals [11], see Table 2.

Direct vs. Indirect	
Direct	Individuals are selected from a parent population using defined criteria
Indirect	Individuals are not considered, but certain statistical methods are applied to analytical values in a laboratory database
A Priori vs. A Posteriori	
A Priori	Individuals are selected for specimen collection and analysis if they fulfil defined inclusion criteria
A Posteriori	Use of an already existing database containing both relevant clinical information and analytical results. Values of individuals meeting defined inclusion criteria are selected.
Random vs. Nonrandom	
Random	Process of selection giving each item (individual or test result) in the parent population an equal chance of being chosen
Nonrandom	Process of selection that does not ensure that each item in the parent population has an equal chance of being chosen

Table 2 Approaches to the selection and identification of reference individuals to establish reference limits. Diagram sourced from Tietz Textbook of Clinical Chemistry and Molecular Diagnostics [12]

It is advised that at least 120 patient are used to calculate 95% reference interval, where the values at 2.5% and 97.5% need to be identified [13]. The assumption is that if the data is presented in a histogram, the data will show a gaussian distribution, but the reference intervals for most biomarkers do not display this distribution and require non-parametric statistical analysis [11].

It was recommended that the direct approach be taken, when establishing reference intervals because the indirect method is impacted by the statistical analysis used, assumptions about the consistency and reliability of the data and the lack of information of any co-morbidities that may exist in this “healthy” population that will impact the final reference interval [14], see Table 3 for examples of exclusion and partitioning criteria.

Exclusion	Partitioning
Age	Age
Alcohol intake	Blood group
Blood donation (recent)	Circadian variation
Drug abuse	Ethnicity
Exercise intensity (recent)	Exercise intensity (recent)
Fasting vs. nonfasting	Fasting vs. nonfasting
Sex	Sex
Hospitalization (recent)	Menstrual cycle (by stage)
Hypertension	
Illness (recent)	
Lactation	
Obesity	Obesity
Occupation	Posture (when sampled)
Oral contraceptives	
Pregnancy	Pregnancy (by stage)
Prescription drugs	Prescription drugs
Recent transfusion	

Table 3 Examples of criteria used to select individuals to develop reference intervals. Diagram sourced from Tietz Textbook of Clinical Chemistry and Molecular Diagnostics [12]

Yet, the direct method is time consuming, labour intensive and costly for laboratories to complete [15]. Consequently, most laboratories do not develop their own reference intervals for each biomarker and instead use the manufacturers' recommended reference intervals. A US survey of 163 laboratories, found that approximately half of the respondents used manufacturers' reference intervals [16]. At UHCW, there is only one biomarker, of the hundreds analysed, that have in-house reference intervals calculated using the indirect method, all other biomarkers have been set up to use either the manufacturers' reference intervals, published data, Pathology Harmony recommendations or values in national/international guidelines.

1.1.1. Clinical decision limits

Clinical decision limits are a different to reference intervals, in that it describes clinical categories that are designed to reflect values in health and disease [17]. These can be designed by assessing biomarkers in healthy individuals and those with disease to set limits that aid diagnosis, monitoring, prognosis and

classifications. Examples of the use of clinical decision limits include those that are described for Diabetes Mellitus diagnosis [18], lipid disorder management [19], renal impairment [20], myocardial infarction [21], and heart failure [22]. Clinical decision limits can include therapeutic ranges, risk calculations as well as actual biomarker values.

1.1.2. Limitations of reference limits/clinical decision points

1.1.2.1. Method bias

The approach above is used to establish the biological variations that impact reference ranges. However, it is also important to take the analytical bias into account. Laboratories seek to find assays that are traceable to a reference material, so that the results produced across laboratories provide the same result, wherever the analysis takes place. This relies on diagnostic companies using these reference materials to develop their biomarkers. In reality, they use different reference materials, reagents, antibodies and chemical reactions in order to provide a result. This results in a bias in the methods against what is considered the "true" values, see Figure 3, which shows the difference in method bias for phosphate presented by the National External Quality Assurance Scheme (NEQAS), Birmingham.

This scheme sends out material to registered laboratories with a set concentration of the biomarker in question and statistically analyses the results against the expected value and across different analytical platforms. The bias needs to be considered when setting reference limits, which complicates the approach of a set reference limit/clinical cut-off that can be applied to an entire population when the analytical methods used in laboratories in the UK vary. The same external quality assurance sample (EQA) can give a significantly different result when measured in lab A on analytical platform A, when compared to lab B on analytical platform B, hence, the approach of using manufacturers' reference limits/clinical cut-offs to take this bias into consideration.

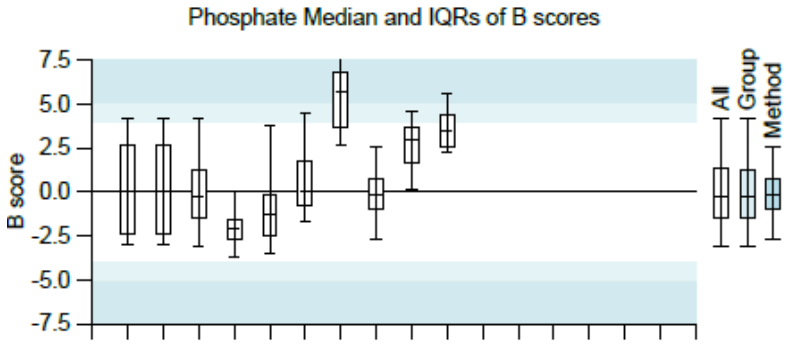

Figure 3 External Quality Assurance performance showing the difference in method bias for phosphate for different analytical platforms (B Score – Bias score). Diagram sourced from Birmingham NEQAS, Clinical Chemistry Distribution 1119. [23]

1.1.2.2. Pre-analytical variables

Pre-analytical variables are another factor that needs to be considered when interpreting biomarker results against reference limits/clinical cut-offs. These variables impact the result obtained and can be differentiated into controllable and uncontrollable variables.

1.1.2.2.1. Uncontrollable variables

Uncontrollable variables include age, gender, race, pregnancy and genetics, see Table 4 for examples of these variables.

Influence	Examples of Analyte Concentrations Changed	Remarks
Age[74,75]	Alkaline phosphatase, LDL-cholesterol, hormones, creatinine.	Provide age-dependent reference intervals
Race[75,76]	Creatine kinase higher in black than in white males. Granulocytes higher in white than in black males. Creatinine higher in black than in white males.	Provide race-specific reference intervals
Gender[74,77]	Alanine aminotransferase, γ-GT, creatinine	Provide gender-specific reference intervals
Pregnancy[25,39]	Triglycerides ↑, homocysteine ↓ during pregnancy	Document months of pregnancy with laboratory results
Altitude[39]	CRP, hemoglobin ↑, transferrin↓	Consider weeks of adaptation, when coming from or going to high altitude

Table 4 Examples on uncontrollable pre-analytical variables. Diagram sourced from Tietz Textbook of Clinical Chemistry and Molecular Diagnostics [24]

1.1.2.2.2. Controllable variables

The area in which laboratory scientists can gave the greatest impact on pre-analytical variables and how to minimise them, are those that can be controlled. These include time of sample collection, dietary factors, medication, sample type, sample tube type, sample stability and sample contamination.

Time of collection can have a direct impact on the interpretation of a biomarker, for instance, cortisol has a diurnal variation, see Figure 4, which shows that cortisol is higher in the morning that in the evening. As such, it is recommended that a sample for cortisol measurement in taken at 9am in the morning for appropriate interpretation against reference intervals.

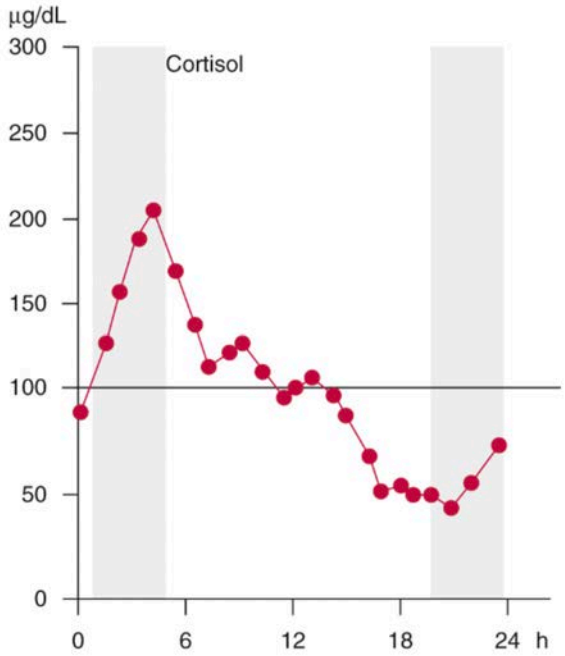

Figure 4 Diurnal variation of cortisol, grey area denotes the sleep period. Diagram sourced from Tietz Textbook of Clinical Chemistry and Molecular Diagnostics [24]

Biomarkers such as LH, FSH, oestradiol and progesterone also need to be measured at a specific time of the menstrual cycles so that the appropriate reference intervals and cut-offs are used to identify causes of amenorrhea or fertility issues, see Figure 5.

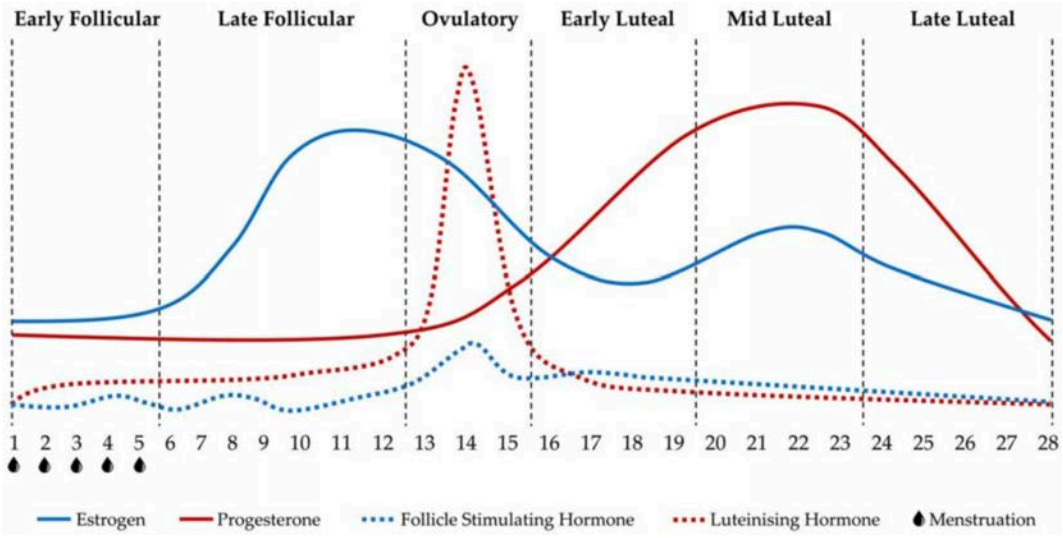

Figure 5 Hormonal changes in a regular menstrual cycle. Diagram sourced from The Impact of Menstrual Cycle Phase on Athletes' Performance: A Narrative Review.[25]

Another important confounding pre-analytical factor on the measurement of biomarkers is the sample collection tube type. The use of certain collection tubes can impact the results obtained, e.g., the use of potassium ethylenediaminetetraacetic acid (EDTA) is an anticoagulant used for the collection of many biomarkers, but it cannot be used for the measurement of potassium, calcium and magnesium [26].

Due to the impact of red cells on the measurement of certain biomarkers, blood samples are collected into gel separator tubes, e.g. potassium, which will leak from the intracellular compartment into plasma if left on cells. However, the use of gel separator gels can also have an impact on the concentrations with certain hydrophobic compounds binding to the gel [27]. As such, it is important that stability experiments are carried out on any new biomarkers in relation to their clinical use, to ensure that these variables do not significantly impact the concentrations and their clinical application.

1.2 Big data

The importance of using population-based big data for healthcare has been identified as an important tool to provide and plan specific services, detect disease earlier, monitor quality indicators and outcomes, modify treatments and support research and development [28, 29]. However, the use of big data must be used sensitively, regarding data governance, IT compatibility, sources of data that impact biases and their potential errors in collection, correlation does not indicate causality [30]. Another important consideration for big data is how it is interpreted, with such large data set, machine learning and artificial intelligence (AI) are becoming a vital tool [31]. Using big data in machine learning and AI can be supplemented by precision medicine and diagnosis using DNA profiling [32].

The need to provide real time data that is collected and coded in a consistent manner has led to the implementation of the Clinical Practice Research Datalink (CRPD), which collected data from GP practices across the UK [33] and the UK Biobank, which is a large-scale biomedical database and research resource, including genetic and other data from half a million UK participants [34].

The use of machine learning and AI is still in the earlier stages of healthcare evolution as there is a need to validate the approach and application of the outcomes using large data sets, however, the use of clinical data from specific populations is an important inclusion [35].

1.4. Chronic health conditions requiring precision diagnosis

There are several diseases that are recognised as being a health care burden worldwide. These include cardiovascular disease, renal disease, and HIV. The World Health Organisation (WHO) reports that the CVD is the leading cause of death globally, 17.9 million people died from CVD in 2019, which is 32% of the population world-wide, and attribute 38% of premature death to CVD [36].

Chronic kidney disease is also recognised as a leading cause of mortality worldwide [37], with an estimated 843.6 million affected worldwide [38].

HIV is also recognised as a major cause of disease and mortality worldwide [39], though developments in antivirals has improved prognosis, delays in diagnosis are still an issue [40].

Another important healthcare challenge is the detection of prenatal chromosomal abnormalities, such as Down's Syndrome [41], however, the development of non-invasive prenatal diagnosis has improved detection of this condition [42].

It is logical to conclude that early detection, intervention, and prevention can lead to improved outcomes for all the above healthcare conditions. The use of precision diagnosis in these areas would improve detection and intervention.

1.5. Cardiovascular disease and inflammation

Cardiovascular disease is a heterogeneous disease condition where the main underlying cause is atherosclerosis and has been well established that inflammation plays an important role in the development of atherosclerotic lesions, see Figure 6 [43, 44].

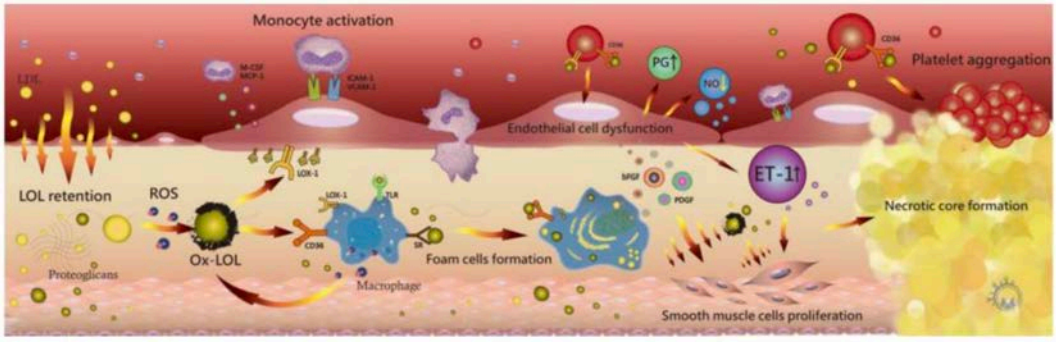

Figure 6 Overview of the inflammatory processes involved in atherosclerosis. Diagram sourced from New Insights into the Role of Inflammation in the Pathogenesis of Atherosclerosis [45]

1.5.1. C-reactive Protein and cardiovascular disease

C-reactive protein is a protein produced by the liver and was first identified in 1930 as a protein precipitated from the cell wall of *Streptococcus pneumoniae* [46]. It has since been shown that it is an acute phase protein that rises 10000-fold in response to an inflammatory state [47]and has been shown to activate the classical complement pathway as part of the immune response [48].

The link between CRP, inflammatory processes and atherosclerosis has been established, see Figure 7.

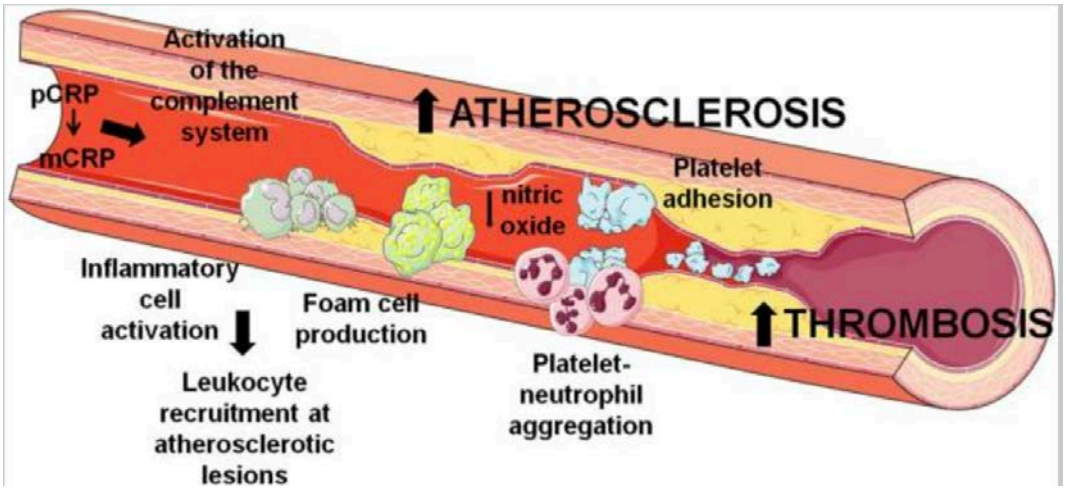

Figure 7 Involvement of CRP in atherosclerosis (pCRP; pentameric CRP, mCRP: monomeric CRP). Diagram sourced from C-reactive protein in Atherothrombosis and Angiogenesis [49]

It would be logical to speculate that inflammatory biomarkers such as C-reactive protein (CRP) can be of use in detecting low-grade inflammation that may lead to adverse events in high-risk patient groups. This could support early identification and intervention, leading to improved outcomes.

1.5.2. Cardiovascular disease and South Asian population

The higher incidence of coronary heart disease in the Indo-Asians when compared to Caucasian populations has been recognized for some time [50-52]. It has also been recognized that traditional markers of cardiovascular risks do not detect the prevalence of CHD in this population [53, 54]. The detection of CHD in this population has become more important to avoid long-term complications

and mortality in this group, particularly as obesity, diabetes mellitus and the metabolic syndrome are increasing in the population in developed countries [55]. The identification of biomarkers that identify this increased risk of CHD would be beneficial and a marker such as CRP has the potential to support this aim.

1.6. Ischaemic heart disease

Ischaemia is a condition that is initiated by the restriction of the blood supply to an organ, followed by the subsequent restoration of perfusion and reoxygenation [56]. Myocardial ischaemia is an important pathophysiological factor in the development of CHD, and its underlying mechanism is well understood [57] , see Figure 8. It has been well established that CHD is prevalent worldwide but also associated with increased mortality [58]. Therefore, early identification of myocardial ischaemia could lead to earlier intervention and the consequent poorer outcomes.

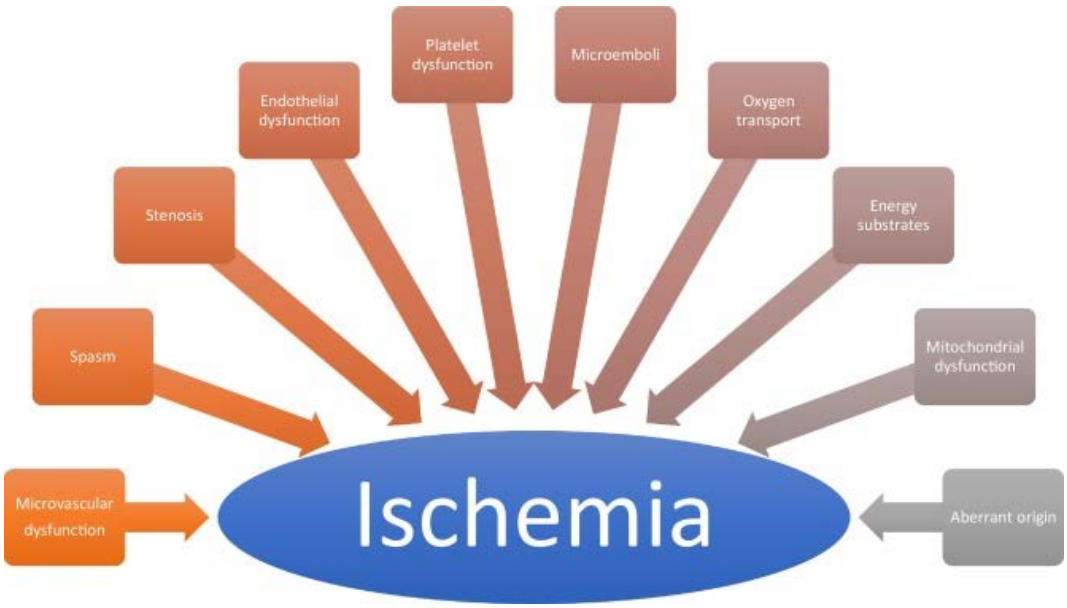

Figure 8 Underlying mechanism identified for ischaemia. Diagram sourced from Myocardial ischemia: From disease to syndrome [59]

1.7. N-terminal B-type Natriuretic Peptide and ischaemic heart disease

N-terminal B-type natriuretic peptide (NT-Pro-BNP) is a natriuretic peptide, which includes the promotion of renal excretion of sodium and water in

response to blood pressure and volume loading [60]. In this setting the ventricular myocardium releases pre-proBNP, which is then cleaved to pro-BNP and then to BNP, which is biologically active, see diagram 9. This leaves the remaining inactive amino-terminal fragment, NT-proBNP, which has a longer half-life [61].

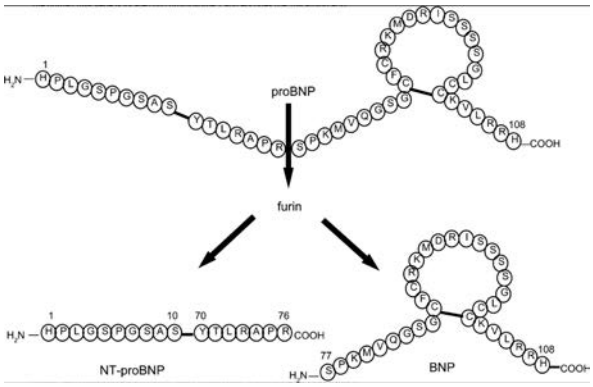

Figure 9 Schematic drawing of proBNP and the cleavage into BNP and NT-proBNP. Diagram sourced from Essential biochemistry and physiology of (NT-pro)BNP [62]

The use of NT-proBNP or BNP is recommended for the diagnosis and assessment of acute and chronic heart failure by the National Institute for Health and Care Excellence (NICE) clinical guidance [22, 63]. However, it has also been shown that NT-proBNP/BNP is an independent predictor of cardiovascular mortality [64, 65] and the measurement is a strong predictor of CHD in patients with stable angina [66].

It has been shown that exercise increases BNP secretion in patients with myocardial ischaemia [67] and that the increase can improve the diagnostic accuracy of the exercise-stress test [67, 68]. However, there is conflicting data on the changes in NT-proBNP when measured as part of an exercise-stress test [69]. More recently, the use of NT-proBNP/BNP to identify the risk of cardiovascular disease is still being shown, yet the measurement of NT-proBNP/BNP is still not in routine use for this purpose.

1.8. Hepcidin

Iron is an important nutrient, but due to the toxicity of free iron and its insolubility in aqueous solutions, and so needs to be bound to a protein or sequestered into cells for long-term storage [70]. For a while the homeostatic control of iron was not well understood until the identification of Hepcidin. Hepcidin is a 25-amino acid peptide hormone produced by the liver [71, 72], which inhibits the export of iron from three compartments, duodenum, recycled iron from macrophages and stored iron in hepatocytes by binding to the ferroportin receptor, see Figure 10 [73].

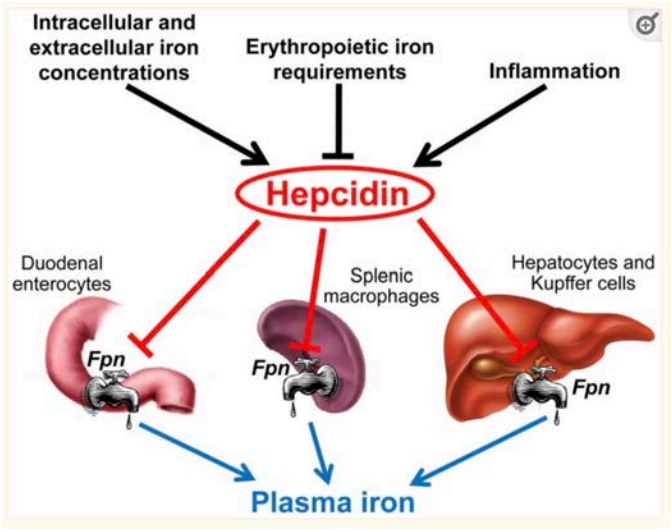

Figure 10. Hepcidin homeostasis (Fpn, ferroportin receptor). Diagram sourced from Hepcidin and Iron Homeostasis [73]

1.9. Hepcidin and the immune system

It is known that iron is required by bacteria, fungi and protozoa for basic metabolic processes [74]. It has been established that any form of immune activation changes the availability of iron, resulting in reduced plasma iron [75]. The changes to iron occur in hours of infection and driven by interleukin-6 (IL-6) and other cytokines through the STAT-3 signalling pathway, see Figure 11 [76]. Therefore, there is an important connection between iron, inflammation and anaemia through the biological actions of hepcidin [77].

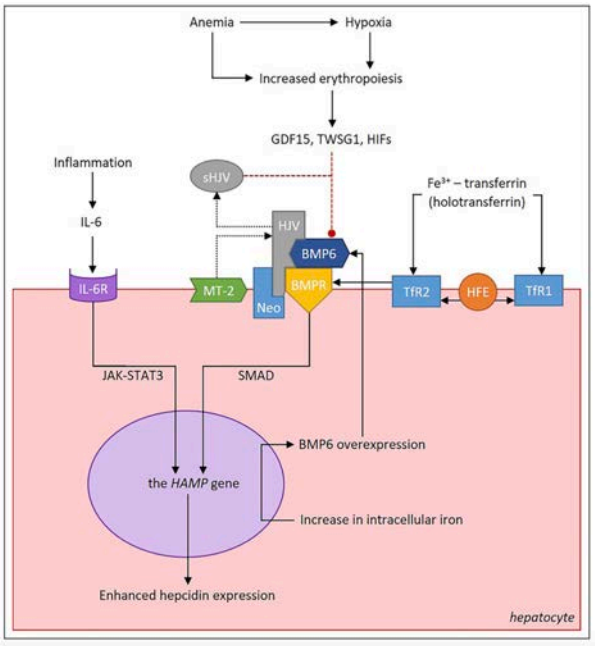

Figure 11. Regulation of hepcidin expression (description in the text). Dashed red arrows with a circular end indicate the process is inhibited. BMP6, bone morphogenetic protein 6; BMPR, bone morphogenetic protein receptor; GDF15, growth/differentiation factor 15; HFE, human homeostatic iron regulator protein; HIFs, hypoxia- inducible factors; HJV, hemojuvelin; IL-6, interleukin 6; interleukin 6 receptor; JAK-STAT3, the JAK-STAT3 pathway; MT-2, matriptase 2; Neo, neogenin; sHJV, soluble hemojuvelin; SMAD, the SMAD pathway; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2; TWSG1, twisted gastrulation 1. Diagram sourced from Role of Hepcidin in Physiology and Pathophysiology. Emerging Experimental and Clinical Evidence [78]

The sequestration of iron into cells protects the human body against infection, and it has been shown in mice there is significantly increased mortality when the hepcidin gene has been knocked out [79]. Conversely, high hepcidin is not always of benefit, with iron sequestered in macrophages encourages intracellular bacteria [80, 81].

1.10. Hepcidin and HIV, Hepatitis B Virus and Hepatitis C Virus

It has been shown that hepcidin has an important role in the pathophysiology of HIV-1, HBV and HCV [82-84]. Viruses need iron for replication, cellular metabolism, and viral protein generation [85].

In patients with HIV-1, anaemia is common and is predictive of increased morbidity and mortality independent of other markers [86, 87]. By sequestering iron within macrophages due to hepcidin stimulation, there is an inverse

correlation with secondary infections and mortality on HIV-1 [88]. Several studies have shown macrophage iron loading in patients who are HIV-1 positive and highlighted the positive correlation between the degree of iron loading in bone marrow macrophages and mortality [88]. It is hypothesised that the macrophages may contain HIV-1 virus that is particularly drug resistant [89] and cause secondary infections, such as, *M.tuberculosis*, which target macrophages [90]. This creates a climate of intracellular iron being used by HIV-1 for replication and secondary infections. There is lack of data on the relationship with hepcidin concentrations in the early stages of HIV-1 infection [91].

There is a known association with iron overload in the liver and HCV and increased mortality and morbidity [85]. It has been demonstrated that supressed hepcidin leads to increased iron deposition in patients with HCV [83], which may lead to fibrosis, cirrhosis and hepatocellular carcinoma. Suppressed hepcidin has also been shown in patients with HBV [84], yet little is known about how hepcidin behaves in the acute phase of HCV and HBV infection.

1.11. Chronic kidney disease and estimated glomerular filtration rate

The measurement of glomerular filtration rate (GFR) is an important tool to identify renal impairment, which is a known health care burden associated with poor outcomes if not treated appropriately [92]. The gold standard to measure GFR is the inulin clearance test, however, this is a time-consuming process, requiring patients to attend hospital and alternatives available include those using radioactive tracers [93]. Due to the incidence and prevalence of renal impairment in the population, this method is not suitable for larger scale healthcare monitoring of GFR. Therefore, a surrogate measure of GFR has been established for several decades, which is to use serum creatinine measurement and include the result in a calculation to estimate the GFR.

The estimated glomerular filtration rate (eGFR) uses age, gender and creatinine in a formula to calculate the filtration rate. At the time of this study, the MDRD formula was being used [94]. The eGFR formula is used to identify the renal function as a routine part of blood testing in primary and secondary care. The results of eGFR are used to categorise patients against the chronic kidney disease (CKD) stages from NICE guidelines, which provides guidance on interventions, monitoring, referral and treatment [95].

Data has shown that those patients with CKD and a lower eGFR have poorer outcomes [19], and also that earlier intervention in those with progressive CKD can be beneficial to the patient by delaying end-stage renal disease [96, 97]. There has also been evidence that delays in intervention in this patient cohort is linked to poorer outcomes [98].

1.12. Cell-free plasma DNA

Cell-free plasma DNA (cfp) was first identified in 1948 [99], but its potential use to detect disease was later suggested for clinical conditions such as systemic lupus erythematosus [100], malignant disease [101, 102] and prenatal diagnosis of genetic disorders [103, 104]. Then potential to use cfp DNA in numerous applications to detect disease, initiated an entire new area of research and the potential application at various stages of disease screening, detection, diagnosis, treatment, and monitoring, see Figure 12.

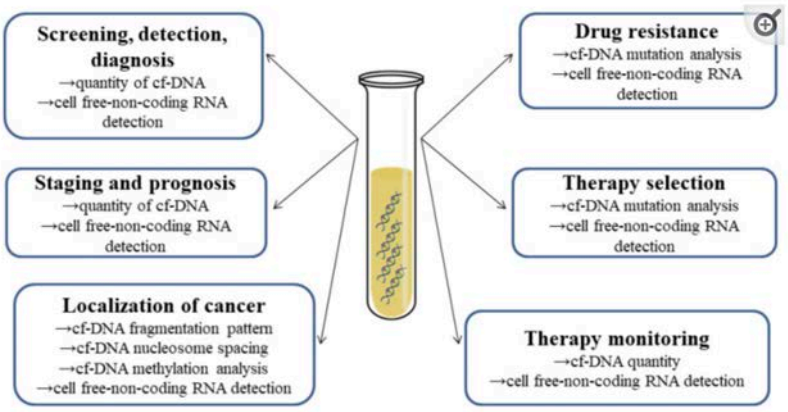

Figure 12 Utilisation of cell-free DNA in diagnostics and treatment. Diagram sourced from Circulating Cell-Free Nucleic Acids: Main Characteristics and Clinical Application [105]

Detection and quantification of circulating cfp DNA is still a challenge and only a few cfp based tests are approved for clinical use [105]. However, there is still great potential in this area, once there is greater understanding of the types of circulating cfp DNA and their biology in specific diseases [106]

1.13. Adrenal insufficiency

Adrenal insufficiency (AI) is a medical important condition, where the adrenal gland is unable to produce cortisol and is associated with poorer outcomes and mortality [107]. The presentation of adrenal insufficiency commonly include weight loss, anorexia, nausea, vomiting, lethargy and fatigue and in primary adrenal failure, there is also postural hypotension, muscle cramps and abdominal discomfort due to mineralocorticoid insufficiency [108]. Due to the insidious course of the disease, patients may not be diagnosed until they present with hypotension, tachycardia and hypovolaemia with associated disorientation or impaired consciousness, known as adrenal crisis [109], which is considered a medical emergency. The diagnosis of AI is important, to enable treatment to begin and prevent patients presenting with adrenal crisis and this has been supported by national guidelines [110].

The gold-standard test for diagnosing AI is the insulin stress test, however, it is considered too harmful and has been replaced by the short synacthen test (SST). This test includes the taking of a sample at baseline for cortisol, administration of synthetic adrenocorticotrophic hormone (ACTH) called synacthen. A second sample is taken after 30 mins for cortisol (and occasionally at 60 mins). The accepted interpretation of a "normal" response to synacthen is a basal concentration of >200 nmol/L, 30 min cortisol of >550 nmol/L, which were developed from fluorometric methods, that have been concluded to produce higher results than the current cortisol methods [111-113]. It is important that the application and use of this test is standardised but also considers any pre-analytical and analytical considerations into account when interpreting the results to ensure appropriate diagnosis.

1.14. Precision diagnosis

The concept of precision diagnosis came as part of the initiative by President Obama in 2015, where precision medicine was launched. The approach to patient care is more bespoke and individual to the patient and takes into consideration genetic, lifestyle and social economic background [114]. This approach opposes the methods and practices that are mentioned earlier in this thesis, where data collection is obtained from a heterogeneous population with some aspect of exclusion and inclusion criteria applied.

There has been acknowledgement for some time that the biomarkers cannot always be transferred from one population in which the reference intervals and clinical decision limits are developed, to another with a uniquely different population that reflects a variable age, gender, ethnic groups and social economic background [115, 116]. This has meant that the specificity and sensitivity of the biomarkers can vary and the ability to detect disease in these groups is negatively impacted. Additionally, current biomarker are not assessed on an individual basis, e.g. eGFR not being monitored closely enough in an individual to detect slow deterioration to end-stage renal failure [117].

The conflict between large population-based interpretation and big data application of biomarkers and the new drive for precision diagnosis is demonstrated in Figure 13. The limitations of precision diagnosis are mostly due to the lack of data and the large individual variations that exist that the current infrastructure in health care cannot support. For instance, laboratory systems can only program one reference interval/clinical cut-off per gender or age group, which will need to be addressed as this area is developed.

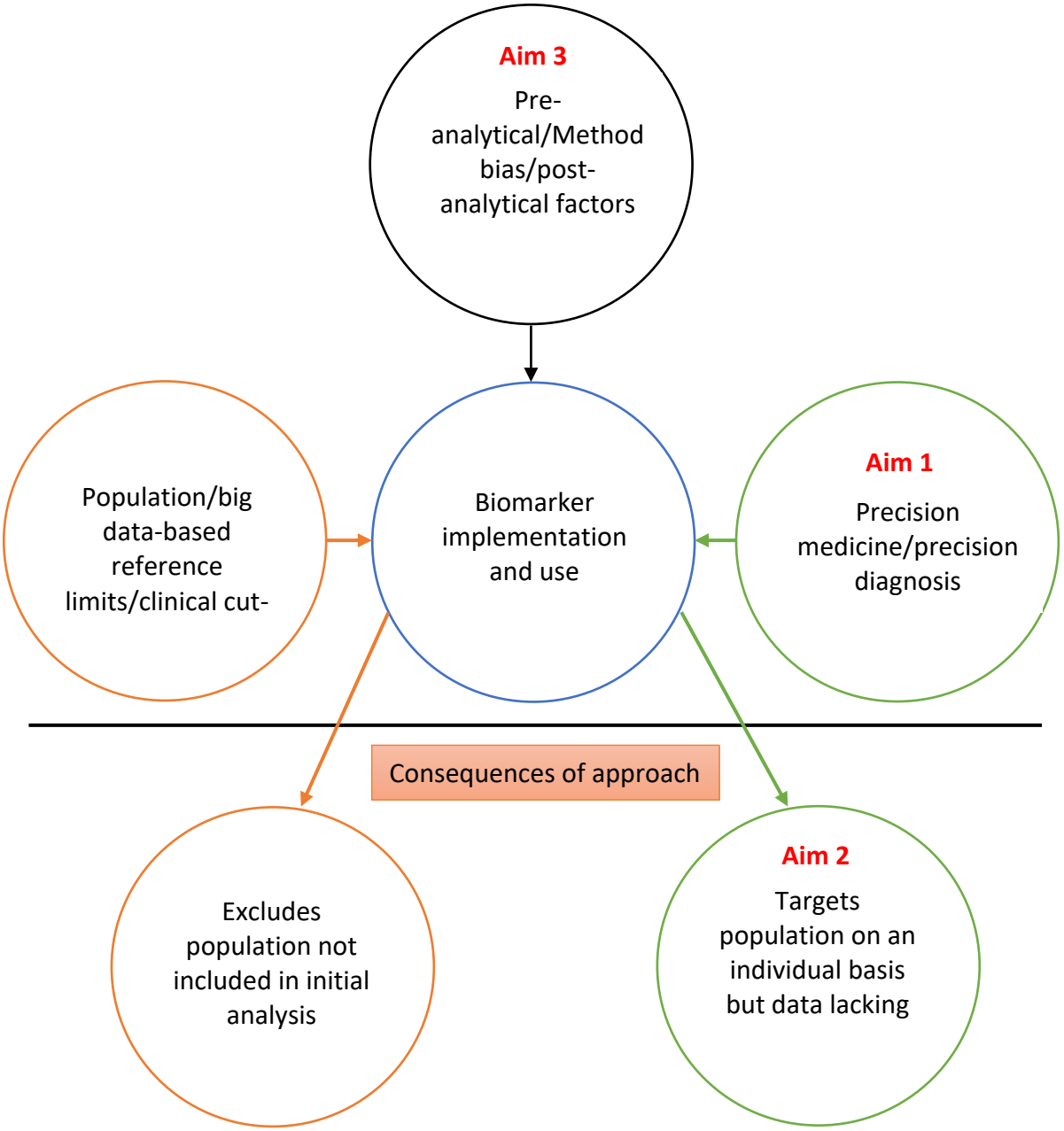

Figure 13 Overview of the dynamics for the implementation and use of biomarkers and the conflicting approaches between population-based reference limits and precision diagnosis. Also marked is where each aim of this thesis contributes to the area.

2. Thesis overview

This thesis seeks to present the optimisation of traditional and new biomarkers to support precision diagnosis by improving detection, monitoring and treatment of patients with new or known chronic conditions. This body of work seeks to address the need for population and individual specific biomarker data to improve outcomes in patients that is contrary to the traditional large population-based application of biomarkers.

The aim of each paper and its contribution to the field is stated below. Each publication is summarised with the contribution I made to each one.

3. Aims

Aim 1. Increased detection of disease in high-risk populations (Paper 1 and 2)

- Paper 1 aims to identify a novel biomarker at the time, high-sensitive C-reactive protein (hsCRP), as a potentially superior cardiovascular marker to detect CHD in the South Asian population.
- Paper 2 aimed to show the clinical application of a novel biomarker, NT-pro-Brain Natriuretic Peptide (NT-proBNP), in identifying patients with ischemic heart disease (IHD) when used with the exercise stress test. This study aimed to show its clinical usefulness and potential as a diagnostic biomarker to improve detection of CHD.

Aim 2. Improved detection of poor prognosis in individuals with chronic disease (Paper 3 and 4)

- Paper 3 aims to identify the relationship between iron status, hepcidin and viral infections such as HIV-1 and Hepatitis B and C, which may indicate later viral load and poorer outcomes.
- Paper 4 aimed to improve the outcomes of patient with chronic kidney disease (CKD), who are being monitored by primary care. This study showed the ease and value of presenting eGFR in graphical form to highlight patients with deteriorating renal function to ensure earlier intervention and preventing/delaying end stage renal failure.

Aim 3. Optimising pre- and post-analytical use of biomarkers for their clinical application (Paper 5, 6 and 7)

- Paper 5 The optimization of cell-free DNA testing by identifying the conservation of methylation patterns of GNAS in circulating blood for its potential use in pre-natal diagnosis.
- Paper 6 Establishing the stability of C-reactive protein (CRP) for its use as a cardiac biomarker to ensure accuracy of measurement.

- Paper 7 Identifying the national use and interpretation of the short synacthen tests (SST) to identify variations in pre-analytical and analytical factors that can impact interpretation of results.

4. Increased detection of disease in high-risk populations

Paper 1

Ethnic variation in C-reactive protein: UK resident Indo-Asians compared with Caucasians, *J Cardiovasc Risk.* 9(3):139-41.

Chatha K, Anderson NR, Gama R, (2002)

Citations (22)

IF: Journal now called the European Journal of Preventative Cardiology – impact factor 5.640

Having recognised the evidence regarding the correlation between CRP and CHD the evidence of the use of CRP in an ethnic diverse group has been conflicting [118, 119].

The routine investigation of CHD risk, at the time, was to calculate the 10-year risk of CHD using the Framingham Risk equation. To complete this calculation several parameters and biomarkers must be used. The Framingham Risk equation uses age, sex, systolic blood pressure (BP), serum high-density lipoprotein (HDL) cholesterol, smoking, diabetes mellitus and left ventricular hypertrophy to calculate the 10-year risk score of CHD.

It was hypothesized in this study that the CRP may correlate with higher risk scores and provide an independent biomarker for CHD in the Indo-Asian population due to the link between inflammation and CHD.

Blood samples collected for the cardiovascular biomarkers were collected in General Practitioner (GP) practices and sent to the laboratory for analysis. A label in placed on the form detailing all the other parameters required for the Framingham Risk equation. These values were copied into the laboratory information system (LIMS), and when the biochemistry biomarkers were analysed, the LIMS calculates the 10-year risk score.

I would ensure that all analysis of tests was complete and aliquot off the remaining serum for CRP analysis. I also recorded the medical history of the patient that was relevant to any exclusion criteria and data interpretation.

Patients were excluded from further analysis if they were <40 years of age, and >70 years of age, known to have arterial disease, diabetes mellitus, smoking, lipid treatment and CRP results >6mg/L. This meant that 191 patients were eligible for statistical analysis. This group was further classified into 102 male (39 Indo-Asian) and 89 female (31 Indo-Asian).

I analysed all samples on the high-sensitive CRP immunoassay method on the DPC Immulite after calibration and quality control checks. The CRP results were added to the Framingham Risk equation data and I completed statistical analysis on this combined data.

The results showed that there was no significant difference in CRP results between the Indo-Asian and Caucasian groups, see table 5; however, there was significantly lower HDL cholesterol in the Indo-Asian female group when compared to the Caucasian female group ($p<0.005$). CRP was significantly correlated with higher risk scores and with Total:HDL cholesterol ratio and negatively with HDL cholesterol ($p<0.005$). In the female group, CRP was significantly correlated to increasing age ($p<0.005$).

CRP and cardiovascular risk factors in: (a) Indo-Asian and Caucasian men

	Indo-Asian	Caucasian	P value
Number	39	63	
Age (years)	52.1 (7.0)	55.2 (7.7)	0.0613
CRP (mg/l)	1.77 (1.46)	1.94 (1.45)	0.5779
Cholesterol (mmol/l)	5.85 (0.89)	5.79 (0.99)	0.7666
HDL cholesterol (mmol/l)	1.32 (0.32)	1.36 (0.37)	0.5475
Total:HDL Cholesterol ratio	4.70 (1.25)	4.50 (1.20)	0.4214
Systolic BP(mmHg)	136.2 (17.1)	139.8 (21.7)	0.3811
% 10 year CHD risk	11.1 (6.4)	11.9 (6.5)	0.5842

(b) Indo-Asian and Caucasian women

	Indo-Asians	Caucasian	P value
Number	31	58	
Age (years)	53.3 (9.4)	56.1 (7.9)	0.1475
CRP (mg/l)	2.29 (1.52)	2.23 (1.54)	0.8607
Cholesterol (mmol/l)	5.58 (1.20)	5.93 (0.84)	0.1064
HDL cholesterol (mmol/l)	1.39 (0.45)	1.67 (0.42)	0.0040
Total:HDL Cholesterol ratio	4.26 (1.23)	3.76 (1.12)	0.0542
Systolic BP(mmHg)	136.5 (16.4)	136.2 (22.8)	0.9529
% 10 year CHD risk	6.6 (5.4)	6.1 (4.3)	0.6164

Results are means (SD).

Table 5 Comparison between CRP and cardiovascular risks between Indo-Asian and Caucasian group

This study contributed to the published data at the time, which was not conclusive regarding the role of CRP and cardiovascular risk in specific populations. This study has contributed to the publications of others working in this field, where the role of CRP and inflammatory markers with CHD in Indo-Asian and other populations have been discussed and investigated, which have further contributed to this area [120-122]. Some of which have shown that CRP is increased in Indo-Asian populations.

In conclusion, this study contributed to a debate at the time that measuring CRP at very low concentrations could be used detect CHD at an earlier time in those at higher risk. Though there was not a significant relationship between CRP concentrations and high-risk scores, this was still an important finding to add to the very few studies in this area. It must be noted that CRP is not used as part of a risk score calculation for CHD risk scores and has not been conclusively proven to improve disease in a high-risk patient group. However, the Framingham Risk equation has been replaced by the QRISK calculation for routine use, due the

improved performance of this calculation by taking more risk factors into account.

More studies have shown that raised CRP is linked to metabolic syndrome and insulin resistance in South Asians [123],which are known to increase risk of CHD [124]. However, another study found that CRP was not significantly different between Caucasian and South Asian groups with metabolic syndrome [125]. Despite this evidence hsCRP is not currently being used to identify increased risk in this population.

Paper 2

B-type natriuretic peptide in reversible myocardial ischaemia, *Clin Pathol.* 59(11):1216-7.

Chatha K, Alsoud M, Griffiths MJ, Elfatih A, Abozguia K, Horton RC, Dunmore SJ, Gama R, (2006)

Citations (10)

IF: 2.894

To identify patients with underlying myocardial ischaemia, an exercise stress test (ETT) is used, which includes the use of a 12-lead ECG and is well established test [126]. There is no biomarker used in this test and is based on the clinician interpreting the results.

B-type natriuretic peptide (BNP) and N-terminal pro-B-type natriuretic peptide (NT-proBNP), at the time of this study, was a novel cardiac biomarker. It has been proven that these biomarkers were positively associated with CHD [127, 128] and it had been suggested that BNP and NT-proBNP could be raised in transient myocardial ischaemia, however, there was no consensus view on this at the time of this study [129, 130].

Patients who were undergoing an exercise stress test (ETT) to detect reversible myocardial ischaemia were consented to take bloods for NT-proBNP before and

after the test by the clinical team. The clinical team would contact me to let me know that the bloods would be arriving from consented patients. I would take receipt of the samples, centrifuge them and aliquot them for analysis. I recorded the relevant past medical history, as we excluded those with other cardiac conditions/treatment, renal dysfunction and hypertension from further analysis. Based on the exclusion criteria, 14 positive ETT and 45 negative ETT patient samples were analysed for NT-proBNP. I completed the analysis of these samples on the Roche Elecsys analysers and completed the statistical analysis of the data. The results showed that NT-proBNP was higher before and after an ETT in those patients with a positive ETT ($p<0.05$) than those with a negative ETT, see Table 6 below. NT-proBNP did rise in both groups after ETT ($p<0.05$) but there was no significant difference in the increase between the positive and negative ETT patient group.

	Positive exercise ECG	Negative exercise ECG	p Value
Number	14	45	
Sex, M/F	11/3	16/29	0.030
Age (years)	60.9 (11.8)	57.2 (13.8)	0.340
Systolic BP (mm Hg)	145.9 (14.4)	137.0 (19.8)	0.106
Diastolic BP (mm Hg)	84.1 (12.7)	83.9 (10.6)	0.997
Resting heart rate (beats/min)	75.5 (12.3)	75.4 (11.6)	0.999
Maximum heart rate (beats/min)	141.6 (16.9)	149.1 (22.7)	0.266
Maximum metabolic equivalents	5.97 (0.65)	9.17 (3.30)	0.002
Time exercised (min)	4.1 (2.85)	9.1 (3.2)	0.004
Pre-exercise NT-proBNP (ng/l)	71.4 (41.2)	54.0 (61.2)	0.013
Post-exercise NT-proBNP (ng/l)	76.8 (44.0)*	60.1 (69.0)*	0.020
Increase in NT-proBNP (ng/l)	4.7 (4.2)	6.2 (8.6)	0.619

BP, blood pressure; ECG, electrocardiogram; F, female; M, male; NT-proBNP, N-terminal proB-Type natriuretic peptide.
*p<0.001 compared with pre-exercise.
Results are raw pretransformed data expressed as mean (SD).

Table 6 Comparison of the relevant factors between the exercise tolerance test cohorts.

This study supported the very few studies at the time [129], which also showed higher BNP values in those with positive myocardial ischaemia compared to those without. However, this study did not support other data that showed a significant incremental increase in those with myocardial ischaemia when compared to the control group [67, 131, 132], and one study that showed no difference between the two groups [130]. This difference may be due to the differences in the study group, length of exercise and differences in establishing myocardial ischaemia. Additionally, the positive ETT cohort had a lower female ratio when compared to the negative ETT cohort.

In conclusion, our study shows that baseline NT-proBNP is raised in patients with symptomatic CHD and may support the investigation of this disease. This study contributes to the data in this area and provides evidence that NT-proBNP has value in supporting the diagnosing myocardial ischaemia to supplement the ETT, supporting the aim of improving the accuracy of detecting disease in a high-risk group. Currently, NT-proBNP is being used in a similar manner to detect chronic heart failure [22], and there is scope to expand the use of this biomarker in diagnosing CHD at an earlier stage based on the data that this study has contributed to.

5. Improved detection of poor prognosis in individuals with chronic disease

Paper 3

Distinct patterns of hepcidin and iron regulation during HIV-1, HBV, and HCV infections, *Proc Natl Acad Sci USA*. 2014;111(33):12187-92.

Armitage AE, Stacey AR, Giannoulatou E, Marshall E, Sturges P, **Chatha K**, Smith NMG, Huang XJ, Xu XN, Pasricha SR, Li N, Wu H, Webster C, Prentice AM, Pellegrino P, Williams I, Norris PJ, Drakesmith H, Borrow P, (2014)

Citation (80)

IF: 9.412

This study aimed to discover the patterns of hepcidin concentrations during the acute and chronic stages of HIV-1 infection and then compare the kinetics between HIV-1, HCV and HBV infections. Additionally, HIV-1 replication requires cellular iron [85], and it was considered whether there was a relationship between hepcidin and HIV-1 replication.

Twelve patients, who were plasmapheresis donors, had sequential samples taken to measure hepcidin and other iron biomarkers, who were later diagnosed with HIV-1 to assess hepcidin in the acute phase. Additionally, another 21 patients were recruited that had been recently infected with HIV-1 to assess the transition from acute to chronic infection. Cytokines were also measured at the time due their role in hepcidin up-regulation.

In this study, I analysed all the samples for hsCRP, ferritin, iron and UIBC so that these biomarkers could be used to interpret the hepcidin and cytokine concentrations obtained from the patients.

Results of the study showed that in the acute phase, hepcidin rose significantly, peaking around day 11, which occurred soon after the elevation on acute phase proteins. In those transitioning from acute to chronic infection, hepcidin remained elevated at an early point before stabilising when the set-point

viraemia became established. This study also showed a clear associated of high set-point hepcidin with high set-point viral load, which a known factor for time to acquired immunodeficiency syndrome (AIDS) and death [133]. This study also confirmed that the same up regulation of hepcidin is not seen in HCV and HBV.

This study summarised the implications of this data and surmised that hepcidin upregulation due to inflammation, favours HIV-1 replication, explaining the increased viral load. Hepcidin also promotes HIV-1 associated anaemia, which as discussed earlier is an important comorbidity factor during HIV-1 infections. Additionally, iron loading in macrophages caused by hepcidin inhibiting macrophage functions, allowing macrophage-tropic infections to take hold.

This research contributes considerably to this area, as the unique hepcidin changes during the acute phase on an infection are consistent with later iron distribution patterns and in turn iron distribution may contribute to subsequent disease progression. Which may explain poorer outcomes in some patients with HIV-1. As such, this biomarker and iron patterns may have potential to identify those with poorer outcomes in patients with HIV-1, yet there is more research required in this area before it can be used routinely.

Paper 4

Kennedy DM, **Chatha K**, Rayner HC, (2013), Laboratory database population surveillance to improve detection of progressive chronic kidney disease, *J Ren Care.* 39 Suppl 2:23-9.

Citations (18)

IF: 1.216

This study sought to develop and validate a software programme that was interfaced with the LIMS to present a cumulative five-year summary of eGFR results, which would ensure improved detection of a slow deterioration of renal function. This study was designed to firstly retrospectively review the graphs that had been previously reported by the renal consultant to identify the long-term outcomes of patients, and secondly to establish a process the generated graphs are reviewed by a clinical scientist, who then selects those that need clinical intervention based on the trends observed. This would also allow the clinical scientists to develop the software.

In this study, the software would select a particular cohort of patients from the LIMS systems, I would then review the graphs on a weekly basis and select those that I interpreted as either “high” or intermediate”, risk using my own visual interpretation of the graphs. There is no algorithm or statistical analysis used to interpret these graphs, due to the need to cascade the process to other laboratories. The graphs that were selected to these two groups were then printed with a letter giving advice on intervention. I would then post these reports to the relevant GP practice. I also reviewed all the previous graphs as part of the retrospective audit of this study. The retrospective audit was completed for two reasons, one was to establish the long-term outcomes of this intervention, which had been previously completed by the renal consultant, the second was to validate that a clinical scientist could interpret the graphs in consensus with the renal consultant. This would then allow this process to be cascaded to all other laboratories and permit clinical scientists to interpret the

graphs without missing significant renal decline that would impact the clinical outcome of the patient.

The results of the retrospective audit showed that those patients we identified as high risk had a significantly higher mortality rate after 3.5 years and significantly higher chance of requiring dialysis after 4.5 years of follow-up. Five patients who had a significant reduction in their eGFR had no evidence of secondary care referral. Part of this study included a questionnaire to GPs who reported a positive response to this new service (73%).

This study was considered a useful tool to identify and intervene progressively deteriorating renal function and was successful in receiving funding from the SHINE project, The Health Foundation. This allowed the team to roll-out the software and processes to other hospital sites. I completed several talks and presentations promoting this study at The Health Foundation events and met with other clinical scientists to observe the process. The impact of this initial project led to a national quality improvement initiative to spread eGFR graph surveillance for early identification, support and treatment of people with progressive chronic kidney disease (ASSIST-CKD) and further publications have come from this novel way of providing biomarker results for high-risk patients [134-136].

In conclusion, this study has had a significant impact in providing evidence to support a national roll-out of a service that has been designed to use biomarkers in a novel and specific manner to detect and prevent end-stage renal disease in high-risk patients with CKD. In this case, intervening in this specific population supports the move towards precision diagnosis as opposed to the large population-based screening currently employed in most primary and secondary health care.

6. Optimising pre- and post-analytical use of biomarkers for their clinical application

Paper 5

Puszyk WM, **Chatha K**, Elsenheimer S, Crea F, Old RW, Methylation of the imprinted GNAS1 gene in cell-free plasma DNA: equal steady-state quantities of methylated and unmethylated DNA in plasma, *Clin Chim Acta*. 400(1-2):107-10.

Citation (7)

IF: 2.615

At the time of this study, the use of cell-free plasma (cfp) DNA was being suggested a novel biomarker in the application for cancer detection and pre-natal diagnosis of foetal aneuploidy such as Down's syndrome to improve diagnostic accuracy.

The current screening for Down's syndrome, includes the measurement of a specific set of biomarkers [137]. There are limitations to the screening programme as there is still false positives and false negative produced from the interpretation of the biomarkers that can have a significant negative impact on parents and the foetus. Improving cancer diagnosis has also been recognised as an important health care problem [138].

One of the approaches being developed to improve detection of these medical conditions was to quantify of methylation patterns of DNA derived from oncogenes and tumour suppressor genes [139] and also in pregnancy [140]. It had already been established that a proportion of cfp DNA in maternal blood during pregnancy is from the foetus [141, 142].

At the time of this study, the clearance of the cfp DNA due to methylation patterns was not clear. It had already been established that certain unmethylated CpG (Cytosine-guanine) dinucleotides stimulate an immune response [143, 144]. Therefore, it could be surmised that the methylation patterns of cfp DNA may be degraded. In order to quantify methylation patterns

in cfp DNA, it is important to identify if the methylation patten is maintained in steady state, so that accurate interpretation of the biomarker results is completed. The aim of this study was to confirm if the methylation pattern was maintained in plasma when compared to cellular DNA.

In this study, I extracted the cell-free DNA cfp DNA and completed the bisulfite conversion, cloning and sequencing of GNAS-1.

We were able to show that the paternal and maternal methylated patters could be identified and importantly that the methylation patterns were conserved in cfp DNA in the 7 donors, see Figure 8.

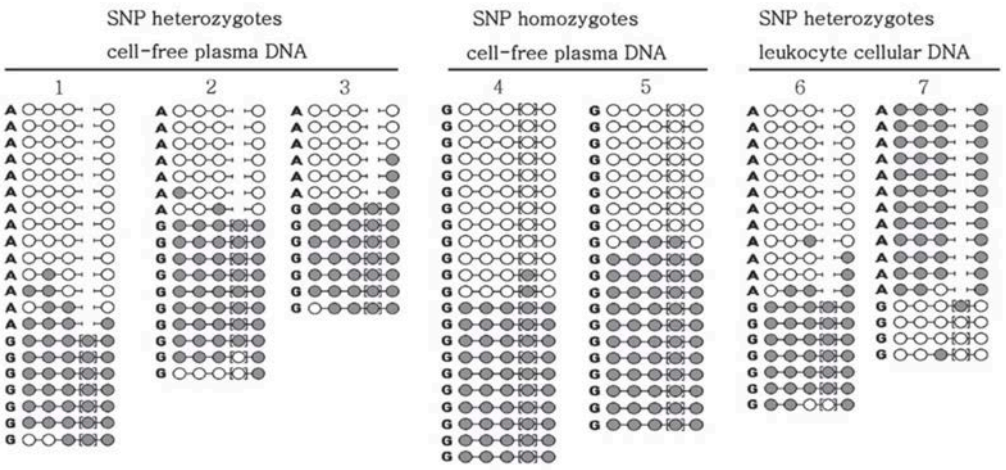

Figure 14 Methylation status of four or five CpG sites in seven patients. Each methylation status is sequenced by an open (unmethylated) or closed (methylated) circle.

This study showed for the first time steady-state methylation patterns were confirmed and that the use of cfp DNA methylation patterns are a viable method for use in cancer, pre-natal diagnosis or any other condition that methylation patterns in cfp DNA for diagnosis. Therefore, improving the diagnostic accuracy for the potential use in cancer diagnosis and foetal screening for high-risk pregnancies. This study has added to the data that supports the use of quantifying methylation in cfp DNA and support precision diagnosis.

Paper 6

Anderson NR, **Chatha K**, Holland MR, Gama R, (2003), Effect of sample tube type and time to separation on in vitro levels of C-reactive protein, *Br J Biomed Sci.* 60(3):164-5.

Citation: 6

IF: 2.712

As it has been discussed earlier in this thesis, high-sensitive CRP has been shown to have a potential role in identifying patients at higher risk of CHD. Due to the low concentrations that need to be measured in this clinical context, it is important to know any pre-analytical variables that may impact the accuracy of measurement. As such, it is vital to establish the long-term stability of CRP in various sample collection tubes and the time to centrifugation that may cause deterioration in the quality of the measurement. At the time of this study, this evidence had not been established for the stability of hsCRP.

The study was designed to collect blood from each consented patient into three different sample collection tubes, 10ml plain glass tube, a 2.7ml EDTA tube and a 4.2ml gel tube. Samples were centrifuged at the following times after collection; 20 min (baseline), 1, 2, 4, 8 and 24 hours. I analysed all samples for hsCRP on the DPC Immulite.

The data showed there was significant differences between hsCRP results in samples collected at 20 mins ($p<0.05$), see table 7 below, using two-way ANOVA. High-sensitive CRP concentrations were significantly higher in gel tubes ($p<0.05$) when compared to glass and ETDA sample tubes.

	Time					
	0.3 h	1 h	2 h	4 h	8 h	24 h
Tube A (Plain)	17.0(17.3)	16.6(16.8)	17.2(17.9)	17.4(17.7)	17.7(18.1)	17.8(17.6)
	A/C*					
Tube B (EDTA)	16.8(17.1)	17.2(17.8)	16.7(16.4)	16.0(16.1)	15.9(16.6)	17.2(18.2)
	B/C*					
Tube C (Gel)	18.5(18.3)	17.6(17.7)	17.4(17.3)	17.5(17.9)	16.6(16.3)*	16.7(16.9)*

Results are mean (Standard Deviation)
Within sample tube C (Gel), compared to 0.3 h * = $P<0.05$
Between sample tubes. A/C * = $P<0.05$, B/C * = $P<0.05$

Table 7 Mean CRP concentration (mg/L) for each sample against time

There was no significant difference in hsCRP in the glass and ETDA sample tubes over the 24-hour period, however, hsCRP was significantly lower in gel tubes when measured at 8 and 24 hours compared to the baseline concentration ($p<0.05$), which was a decrease of 9.7%.

This study showed that the decrease in the stability of hsCRP may of clinical significance if it being used to support the evaluation of CHD in the general population, and any results obtained from samples that are not centrifuged in the acceptable time limit, must be interpreted with caution. This study provides important pre-analytical factors that can impact precision diagnosis, which must be identified to validate the use of biomarkers before clinical use.

Paper 7

Chatha K, Middle JG, Kilpatrick ES, (2010), National UK audit of the short synacthen test, Ann *Clin Biochem.* 47(Pt 2):158-64.

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Incorrect diagnosis of adrenal insufficiency can be harmful to patients, and the interpretation of the STT using different pre-analytical and analytical considerations need to be considered and there have been recommendations to take a number of factors, including method bias into account when interpreting SST results [113] including the ACB Wales Clinical Biochemistry Audit group findings.

This audit aimed to identify the current practices around the administration of the test, analysis and interpretation of the SST. A web-based format was used, and an invitation to participate was sent to all clinical biochemistry laboratories.

In this audit, I collated all the answers and organised the responses into consistent data that could be presented appropriately. Many of the questions were open text questions, therefore, the responses varied greatly and required careful interpretation so that the results were not biased.

The results of this audit were important as it showed that a universally applied test showed great variability in processes, with 46% of laboratories using 0- and 30-min cortisol sampling and 31% using 0, 30 and 60 mins sampling. Importantly, the cut-offs were the most important clinically, with 65% of laboratories using a cut-off of between 450 and 600 nmol/L as an appropriate response to synacthen, but only 19% took their method bias into account when interpreting the SST, see diagram 8 which displays the bias of the various analytical platforms for cortisol. Additionally, 89% of laboratories use an incremental increase of 200 nmol/L, which has been shown to be unreliable.

True cortisol	All	SF1	AB13	DC11	DC7	COI0	E170	BO5
100	107	101	99	102	90	114	102	102
150	152	138	138	142	131	159	158	158
200	197	175	178	182	173	205	215	215
250	242	212	217	223	215	251	271	271
300	287	250	256	263	256	297	327	327
350	332	287	295	303	298	343	383	384
400	377	324	334	344	339	389	439	440
450	422	361	374	384	381	435	496	497
500	467	398	413	424	423	481	552	553
550	512	435	452	465	464	527	608	609
600	557	472	491	505	506	572	664	666
650	602	509	530	545	548	618	721	722
700	647	546	570	586	589	664	777	778
750	692	583	609	626	631	710	833	835
800	737	621	648	667	673	756	889	891
850	782	658	687	707	714	802	946	948
900	827	695	726	747	756	848	1002	1004
950	872	732	766	788	798	894	1058	1060
1000	917	769	805	828	839	939	1114	1117

AB13, Abbott Architect; CO10, Siemens Centaur; SF1, Beckman Access; DC11, Siemens Immulite 2000/2500; E170, Roche E170 modular; BO5, Roche Elecsys; DC7, Siemens Immulite 1

Figure 15 Cortisol values (nmol/L) for the difference methods calculated using ID-GCMS regression parameters (method mean versus ID-GCMS assigned values) from UK NEQAS data

The audit has shown that taking the method bias and other pre-analytical conditions into consideration, incorrect diagnosis of adrenal insufficiency can be avoided and improve patient outcome by initiating treatment appropriately. These pre-analytical and analytical variations are an important variable that must be considered when developing precision diagnostics.

This audit made an important contribution to this field and has been cited by others to investigate SST interpretation further [145-147] and cited by the Endocrine Society for their Clinical Practice Guidelines for the diagnosis of primary adrenal insufficiency [110]. The inclusion of data, such as this audit, into clinical practice guidelines, is based on the quality of the data published.

There are several approaches that can be used to develop guidelines, one approach is to use consensus development methods, which includes the Delphi process, nominal group technique or a consensus development conference [148].

In the context of this questionnaire, the Delphi method is more appropriate to obtain a clinical practice guideline, which uses a defined round of questionnaires to gather information to gain expert consensus [148, 149]. The advantage of this approach is that it allows contribution from a large, geographically diverse, and anonymous group of experts, avoiding stronger opinions to dominate the conversations [150]

However, a more evidence-based approach was recommended by the Institute of Medicine where they revised clinical practice guidelines as “statements that include recommendations intended to optimize patient care that are informed by a systematic review of evidence and an assessment of the benefits and harms of alternative care options” [151]. In this approach, relevant stakeholders are bought together to critically assess the available data, using techniques such as AGREE II for guidelines [152], AMSTAR for systematic Reviews [153], and the GRADE approach [154].

However, the use of a questionnaire still has benefits in that it provides real-time data, it allows contribution from a larger group and more applicable to the population and it provides large amounts of data in a short period of time at a lower cost [155]. The findings from this questionnaire, could be used as the basis of starting an evidence-based approach as described above.

7. Discussion

The advent of precision medicine and therefore, precision diagnosis has brought into focus the requirement of biomarkers that provide superior accuracy of detecting and managing disease in a diverse population. This thesis first demonstrates the current limitations that stand in the way of the ambitious evolution in healthcare. The thesis presents the current pragmatic processes that identify the usefulness of biomarkers, that are influenced by the limitations of the technology and large population-based data analysis, that either is too heterogeneous or are based on a specific population that cannot be applied universally. The contradicting requirement for more bespoke data for biomarkers to be used in precision diagnosis is an area that needs further development.

This thesis presents the findings of seven publications that aim to meet the requirements of biomarkers to support precision diagnosis to be implemented. The research in these papers cover major health care burdens, worldwide. Therefore, the need to elucidate new approaches to biomarkers an important requirement.

The first aim of this thesis was to demonstrate the ability to identify biomarkers that would detect disease in high-risk patients. The first paper was completed at the time when Ridker *et al*, described a relationship between CRP and risk of cardiovascular disease [156, 157]. This encouraged further research into CRP, particularly high-sensitive CRP, as this is a routinely available test, which had been established for a while. The increased risk of CHD in the South Asian population is well known. Despite Paper 1 showing that hsCRP is higher in patients with an increased risk of CHD risk, but is not significantly raised in the Indo Asian group, the use of hsCRP is still not included in risk score calculation. There have been recommendations to include CRP into risk scores [158] irrespective of the ethnicity of the population due to further evidence demonstrating a link between CRP and increased risk of CHD.

There was still much debate about the clinical role of inflammatory biomarkers and their use in risk assessments, however an important study, called the JUPITER (Justification for the Use of statins in Primary prevention: an Intervention Trial Evaluating Rosuvastatin), showed a 37% reduction in CRP and sharply decreased cardiovascular events and all-cause mortality [159].

There are limitations to this study; there patient group is small, the Indo-Asian population included patients that are from a varied ethnic background, who have been shown to have different risk factors, such as smoking, which is higher in the Bangladeshi population [53]. There are also the differences in the exclusion criteria used in the different studies and we did not have body-mass index (BMI) data which would have been relevant, due to the evidence that obesity is linked increased CRP concentrations [160].

Pearson's correlation was used for the statistical analysis for this data, and there are several limitations to take into consideration; the assumption that the relationship between the two variables is linear, the data is bivariate normal, correlation does not mean causation, there are sufficient data points and that the variability of the two variables may not be equal [161, 162].

The second paper aimed to identify the potential superiority of NT-proBNP as a biomarker to detect IHD. The study found that indeed NT-proBNP was raised in those with a positive ETT compared to those that were negative, which supported the published data [67, 127, 128, 130]. However, the exercise induced increase was similar in both the positive and negative ETT. The differences in this study compared to those previously performed may be due to the differences in study population, processed for completing the ETT, diagnostic criteria, analytical techniques and type-1 and type-2 statistical analysis. Additionally, a power calculation was not performed before the study commenced to ensure it is suitably powered for all outcomes that were measured.

The second aim of this thesis was to show the investigation of biomarkers that improve detection of poor prognosis in patients with chronic disease. This aim is met by the next two papers. Paper three is an important publication that demonstrates that there are distinct patterns of hepcidin regulation in acute and chronic HIV-1, HCV and HBV. The poorer outcomes in patients with HIV-1 and iron abnormalities is an important finding and raises the potential to target hepcidin and iron to improve outcome. However, one study evaluated iron intake in patients with HIV-1, and correlating iron consumption and viral load, where they found that increased iron intake correlated positively with viral load but not CD4+ count [163], this may be due to the complex relationship between viruses and iron as described earlier.

More recently, it has been reported that increased hepcidin is associated with the severity of COVID-19 [164] and the use of hepcidin as a therapeutic target is also being explored, though there is much more research required in this area [165].

Paper four shows a novel use of graphical representation of eGFR results to trigger earlier intervention in patients with CKD. This paper was unique in that it was the first time that software was developed to present eGFR in a cumulative format that would lead to triage and production of correspondence to GP practices to highlight deteriorating renal function and providing advice on interventions to mitigate the poorer outcomes in thus population. This study sought to validate the use of the software and its implementation in routine practice. The clinical impact of such an intervention was identified in an earlier iteration of the software [166], which this study has developed further for national roll-out.

The ASSIST-CKD project is an ongoing randomised control trial which seeks to implement and measure the use of graphs to highlight deteriorating eGFR in at least 19 renal centres in a phase stepped wedge manner [136] based on the evidence from this paper. The findings of this study are yet to be published,

however, a quality review of the 5 centres involved and found that there are several barriers and enablers to the use of these graphs, but ultimately, the intervention has the potential to slow down renal progression of kidney disease due to earlier clinical intervention [135].

The last aim of this thesis was to show contribution to optimising pre- and post-analytical use of biomarkers for their clinical application. This aim is supported by the outcomes of next three papers.

Paper five addressed the pre-analytical considerations required to validate the use of cell-free DNA methylation patterns, for their application for diagnosis of cancers or for prenatal diagnosis of foetal aneuploidy. This study found that the population of cfp DNA contained equal numbers of both maternal and paternal alleles, with their characteristic methylation patterns which corresponded to those found in cellular DNA sources. Therefore, we concluded that methylation patterns have no impact on the representation of the *GNAS1* DNA molecules in plasma, which is important, as we know that the production and clearance of cfp DNA is unknown and that the immune system is known to use methylation as part of its response to stimulus [143, 144]. This study showed that using the quantification of methylation patterns in a reliable measure of the release of a biomarker by a tumour or by the placenta. This need to understand the steady-state of methylated and unmethylated cfp DNA is an important pre-analytical consideration that this paper demonstrates is conserved with regard to *GNAS1*, promoting the use of personalised diagnostic biomarkers to identify pathological conditions, supporting the aims of precision diagnosis.

Prenatal diagnosis has already started to move towards the use of cfp DNA, with the Netherlands using this technology for all pregnant women [167] and there has been a statement released by the American College of Medical Genetics and Genomics suggesting that cfp DNA can replace conventional foetal anomaly screening [168].

The study for paper six, was developed due to the promising data showing that CRP was an important CHD biomarker, as discussed earlier in paper one. However, the pre-analytical stability had not been established at this point, particularly at the low concentrations being suggested as important. This study was completed to address this important knowledge gap. The study showed that CRP declines significantly when collected into gel tubes and this may lead to misclassification of patients when assessing CHD using this test. We stated that caution must be used when assessing CRP concentrations when the sample unseparated from the gel for more than eight hours or more, which is an important pre-analytical consideration.

Paper seven was an audit that sought to identify the pre-analytical, analytical and post-analytical considerations that labs across the UK took into consideration when completing the SST. This study found that there were some significant areas of difference that need to be considered when interpreting SST results. We found that importantly, method bias was not considered by a number of laboratories, advice regarding medication that impacts cortisol measurement was not consistent or comprehensive and other factors such as sex had not been considered even though published data has shown gender differences [113]. This audit recommended the development of national guidelines for short synacthen test interpretation and the process towards that goal has been described earlier. It must be noted that any such guidelines should include more recent developments in the use of salivary cortisol and cortisone, which are easier and more cost-effective procedure as part of an SST [169]. They have also shown superior performance when compared to serum cortisol SSTs [170].

In conclusion, the publications presented in this study support the application of biochemical biomarkers to aid precision diagnosis by addressing three themes, identifying high-risk patients, identifying poor prognosis in patients with chronic disease and identifying pre- and post-analytical variations that can impact biomarker interpretation and use. All the papers here have made an important

contribution to the field they are addressing and, in some cases, supported national projects and guidelines. Ultimately, these publications have collectively contributed to the data that will allow progress towards precision diagnosis.

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